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CEREBROSPINAL FLUID CYTOLOGY IN SCHIZOPHRENIA

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ACADEMIC DISSERTATION

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2. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals I-IV:

I Nikkilä H, Müller K, Ahokas A, Miettinen K, Andersson LC, Rimón R. Abnormal distributions of T-lymphocyte subsets in the cerebrospinal fluid of patients with acute schizophrenia. *Schizophr Res* 1995; 14: 215-221

II Nikkilä, H., Müller, K., Ahokas, A., Miettinen, K., Rimón, R., Andersson, L.C. Accumulation of macrophages in the cerebrospinal fluid of schizophrenic patients during acute psychotic episode. *Am J Psychiatry* 1999; 156:1725-1729

III Nikkilä H, Müller K, Ahokas A, Rimón R, Andersson L. Increased frequency of activated lymphocytes in the cerebrospinal fluid of patients with acute schizophrenia. *Schizophr Res* (*In press*)

IV Nikkilä H, Ahokas A, Wahlbeck K, Rimón R, Andersson L. Neopterin and macrophage inflammatory protein 1 α in the cerebrospinal fluid of schizophrenic patients; no evidence of intrathecal inflammation (*Submitted*)

3. ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
ANOVA	analysis of variance
BBB	blood-brain-barrier
BPRS	Brief Psychiatric Rating Scale
C-C	chemoattractant cytokine
CNS	central nervous system
CSF	cerebrospinal fluid
DSM	Diagnostic and Statistical Manual of Mental Disorders
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
FACS	flow cytometry
fMRI	functional magnetic resonance imaging
HLA	human leukocyte antigen
ICAM-5	telencephalin
IF	interferon
IL	interleukin
MEG	magnetoencephalography
MGG	May-Grünwald-Giemsa
MHC	major histocompatibility complex
MIP-1 α	macrophage inflammatory protein 1 alpha
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
NAA	N-acetylaspartate
PET	positron emission tomography
PB	peripheral blood
RIA	radioimmunoassay
SCID	Structured Clinical Interview for DSM-III-R
SD	standard deviation
SEM	standard error of mean
SPECT	single photon emission computed tomography
TNF	tumor necrosis factor

4. INTRODUCTION

Despite the fact, that schizophrenia has assumed an increasingly important place in the research programs on neurosciences and molecular biology around the world, and also, that a growing body of evidence suggests that this illness is associated with objective changes in the anatomy and function of the brain, the etiology and pathophysiology of this devastating disease has remained unknown. From the very beginning of dementia praecox- and schizophrenia research, right up to our times, there have been findings suggesting that organic factors contribute to the pathogenesis of this disease. When first described in the eighteenth century, schizophrenia was believed to be caused by premature progressive neuronal degeneration (Woods 1998). Before long, infectious diseases were linked to schizophrenia on an epidemiological basis (Bruce and Peebles 1904). The pre-eminence of psychoanalytic and social psychiatry in the mid-decades of the last century was accompanied by a dearth of research interest into the possible neurobiological causes of schizophrenia and other psychoses. In the fifties and sixties, neurotransmitter theories evolved along with the increasing knowledge of dopamine and serotonin and their functions in the central nervous system (Carlsson et al 1958, Carlsson and Lindqvist 1963, Wooley and Shaw 1954). At the same time, occasional findings of virus-like particles and viral antibodies in the body fluids of psychotic patients brought up suggestions of viral or autoimmune involvement in the etiopathogenesis of schizophrenia (Morozov 1954), and yet another era of enthusiasm in psychoneuroimmunology occurred following the description of slow viral diseases such as kuru and Creutzfeldt-Jacob disease in the '70s (Gajdusek et al 1972, Torrey 1988).

Also the neurodevelopmental hypothesis of schizophrenia can be traced back to the first part of the century (Southard 1915), but it has reached its full-scale popularity during the last two decades. This theory states that schizophrenia is based on a disturbance of the orderly development of the brain that takes place long before the symptomatic phase of the illness (Weinberger 1995). The upward trend of the neurodevelopmental hypothesis is primarily based on neuroradiological evidence suggesting brain volume loss already at the onset of schizophrenia, and no significant progression thereafter (Nasrallah et al 1986, Hoffman et al 1991). In the '90s reports of longitudinal neuroimaging studies with findings of progressive brain alterations have arrived on the scene (DeLisi et al 1997, Davis et al 1998) proposing that the static neurodevelopmental model of schizophrenia may be an oversimplification of the puzzle. Recent "two-hit" and "three-hit" pathophysiological models have been making an effort to explain both the contribution of neonatal

and early life complications as well as potential progressive CNS involvement later in the course of the illness (Impagnatiello et al 1998, McCarley et al 1999, Keshavan 1999). These comprehensive hypotheses also attempt to give an alternative explanation for the confusing inconsistency and contradiction that have been characteristic for findings in the field of schizophrenia research. They emphasize the heterogeneity of miscellaneous etiological factors participating in the cascading course of the disease process, instead of purely explaining these discrepancies basing on the heterogeneity of the disease as a nosological entity.

The occasional cytological findings of the peripheral blood investigations of schizophrenic patients have been inconsistent, or they have been based on the repeated trials of a small number of researchers and laboratories (Nikkilä 1997). Therefore, these reports have not offered any progressive channels of research efforts concerning schizophrenia, in spite of their potential importance. Another disadvantage has been the deficiency of cerebrospinal fluid studies, which has left open the question of the potential role of CNS immunocompetent cells in the pathophysiology of schizophrenia. The requirement for cytological investigations within the CNS body compartment in schizophrenia arises from the rapidly increasing knowledge of the central position of mononuclear cells and microglia in the etiopathogenesis of a multiplicity of CNS diseases (Kreutzberg 1996, Fitch and Silver 1997, Persidsky et al 1999).

5. REVIEW OF THE LITERATURE

5.1. Schizophrenia

5.1.1. Genetics

Family, twin and adoption studies on schizophrenia have demonstrated that schizophrenia tends to cluster in families and to have an important genetic component (Tienari et al 1994, Sham et al 1994, Wahlberg et al 1997). Although the schizophrenia susceptibility genes are still to be identified, extensive linkage analyses have recently suggested that these genes may be present on chromosomes 5q, 6p, 8p, 13q, 18p, and 22q. (Shastry 1999, Turecki et al 1997, Bassett and Chow 1999). Incomplete penetrance and environmental forms of phenocopies leave room also for other influences, and schizophrenia is generally thought to arise as a result of interactions between genetic vulnerability and environmental risk factors (Yolken and Torrey 1997, vanOs and Marcelis 1998). Nevertheless, no definite association of particular gene or specific environmental factor with schizophrenia have emerged at present. Genes for differentiation, growth, direction, and repair of axonal connections within the CNS have been suggested for the focus of genetic studies in order to clarify the possible connection between structural brain deviation and genetics in schizophrenia (DeLisi 1999).

5.1.2. Epidemiology

Epidemiologic studies have revealed a predominance of winter or early spring month birth rates among schizophrenics in the northern hemisphere (Torrey et al 1997). There are also indications pointing to the possible effect of socioeconomic class (Harvey et al 1996, Mäkiöyrö et al 1997), ethnic origin and immigration (Harrison et al 1988, Wessely et al 1991, Selten et al 1997), as well as rural vs. urban distribution (Widerlov et al 1997, Mortensen et al 1999) on the incidence of schizophrenia. Epidemiological data from a Finnish birth cohort exposed to a type A2 influenza epidemic in 1957 revealed an elevated risk of adult schizophrenic outcome among those who were in their second trimester of fetal development at the time of exposure (Mednick et al 1988). This

finding has led to numerous studies on the effect of intrauterine infection on the etiopathogenesis of schizophrenia (Adams et al 1993, McGrath and Castle 1995). Although the recent negative findings do not support an association between in-utero exposure to influenza and schizophrenia (Westergaard et al 1999, Grech et al 1997, Selten et al 1999), the question of a possible relationship of infective agents to the early events of neurodevelopmental processes is still unsolved.

5.1.3. Neurochemistry

The majority of neurochemical investigations of schizophrenia have been directed towards monoamine mechanisms (dopamine, noradrenaline, serotonin), amino acid neurotransmitters (gamma-aminobutyric acid, glutamate) and neuropeptides. Emerging knowledge about the interactions between different neurotransmitters in complex neurocircuits has diversified the targets of interest in the neurochemical research on schizophrenia. Particularly dopamine-serotonine (Boulenguez et al 1996; Kapur and Remington 1996; Lieberman et al 1998) and dopamine-glutamate (Fitzgerald et al 1995; Glenthøj and Hemmingsen 1999) interactions have aroused interest.

The dopamine theory of schizophrenia has persisted as the predominant neurochemical hypothesis for three decades. This hypothesis of excessive dopaminergic function in the CNS of schizophrenic patients has received strong support from clinical trials with neuroleptic drugs which inhibit the dopamine-induced stimulation of adenylate cyclase (Clement-Cormier et al 1974), and displace the high-affinity binding of ligands to the dopamine receptor (Seeman et al 1975). The selective therapeutic efficacy of the *cis* optical isomer of the neuroleptic flupenthixol (as compared to *trans* isomer, a less potent inhibitor of adenylate cyclase and a weaker dopamine receptor blocking agent), has supported the view that the neuroleptic effect is based on dopamine effects (Johnstone et al 1978). The knowledge of the anatomy and pharmacology of the subtypes of dopamine receptors has increased during the '90s (Seeman et al 1993, Ricci and Amenta 1994, Hietala and Syvälahti 1996), and the multiplicity of D2-like receptors has been linked to divergent neuroanatomic sites of suspected pathology in schizophrenia (Ariano et al 1992, Seeman 1992). Joyce et al (1997) have hypothesized that D2 receptors in the basal ganglia are the likely site of extrapyramidal symptoms and not antipsychotic effects, and D3 receptors of the mesolimbic system are a likely site of antipsychotic effects, and D2 and D4 receptors in the medial temporal lobe and limbic cortical areas are the sites of additional antipsychotic effects.

The interest in the potential role of serotonin (5-HT) in the pathophysiology of schizophrenia, which emerged originally in 1954 (Wooley and Shaw) has been renewed in recent years. Atypical antipsychotic drugs have proved to be potent 5-HT receptor antagonists and relatively weaker dopamine D2 antagonists (Meltzer 1995), and molecular biological studies have indicated that allelic variations of 5-HT receptor genes may affect susceptibility to schizophrenia and clinical response to atypical antipsychotics (Busatto and Kerwin 1997). In addition to proposals that 5-HT receptors could be critical sites of antipsychotic action, also the impact of serotonin on neurodevelopment has been brought up (Lieberman et al 1998).

The psychotomimetic effects of phencyclidine, a glutamate antagonist, have been taken to suggest that schizophrenia involves reduced brain glutamate function (Castellani et al 1982). The possible importance of glutamate in the pathophysiology of schizophrenia has been suggested on the basis of findings from post mortem studies (Kerwin et al 1990, Deakin 1994). Glutamatergic abnormalities in the anterior temporal cortex of schizophrenic subjects have been suggested to result from the degeneration of fronto-temporal projections (Deakin and Simpson 1997). Also functional imaging studies have provided evidence that glutamate may be involved in schizophrenia, and diminished glutamatergic neurotransmission in the hippocampal glutamate-mediated efferent pathways and cerebral dysfunction in the hippocampus and its target areas have been proposed to explain some of the clinical manifestations of schizophrenia (Tamminga 1999).

5.1.4. Neuroimmunology

The immune system has been a subject of investigation already from the early days of schizophrenia research. In the beginning of the 19th century some clinical observations were made regarding leukocytosis and elevated temperature at the acute phase of dementia praecox (Bruce and Peebles 1904), decline of the total number of white blood cells either at recovery or as the disease became chronic, psychoses associated with some cases of influenza epidemics (Menninger 1928), and schizophrenia-like psychotic symptoms in conditions resulting from encephalitis (McCowan and Cook 1928). These findings led to first immunological theories on schizophrenia, regarding it more or less as a derivative of contagious diseases.

The first reports of the presence of antibrain antibodies in schizophrenic patients came out in 1937 (Lechmann-Facius 1937). Some later studies have supported these findings (Heath and Grupp 1967, DeLisi et al 1985), but also contradictory reports have been published (Schott et al 1998). However,

the autoimmune hypothesis of schizophrenia has survived for decades, not least due to findings of immunoglobulin deviations (DeLisi and Crow 1986, Noy et al 1994), antinuclear antibodies (Ganguli et al 1993), autoimmune-associated B cell subsets (McAllister et al 1989), autoantibodies against heat-shock proteins (Kilidireas et al 1992), HLA antigen associations (Wright et al 1996, Lahdelma et al 1998), and cytokine production abnormalities (Muller et al 1999).

The first reports of nuclear changes in the peripheral blood lymphocytes of schizophrenic patients were published already in 1962 (Kamp). Soon afterwards more morphological aberrations were detected (Fessel and Hirata-Hibi 1963) and later Hirata-Hibi named the predominant atypical lymphocyte type the P cell (Hirata-Hibi et al 1982). The main identifying features of this stimulated cell are its leptochromatic nuclear structure and high cytoplasmic basophilia (Hirata-Hibi and Hayashi 1992). These cells have also been found in some relatives of schizophrenic patients and in patients with myasthenia gravis and rheumatoid arthritis. The significance and specificity of these findings are somewhat ambiguous, but e.g. viral involvement and reaction to mitogenic agents or antigens could be possible explanations for the appearance of these cells. Recently, Kokai et al (1998) have reported significantly elevated numbers of lymphoblasts and activated lymphocytes in psychotic patients by using phase-contrast microscopy combined with a fluorescent staining technique.

McAllister et al (1989) have reported a subgroup of schizophrenic patients who had increased levels of circulating CD5⁺ lymphocytes. These data could support a possible autoimmune cause for schizophrenia, because populations of these cells have been shown to be elevated in autoimmune disorders such as rheumatoid arthritis, progressive systemic sclerosis, and Sjögren's syndrome. Two later studies have investigated CD5⁺ cells in schizophrenia, one with negative findings (Ganguli and Rabin 1993) and the most recent one supporting the original observation (Printz et al 1999).

There are several reports on various alterations in the distribution of lymphocyte subsets in the peripheral blood of schizophrenic patients. The findings from these studies have, however, been contradictory, expressing both high and low CD4⁺/CD8⁺ ratios, or no changes at all (DeLisi and Wyatt 1982, Kaufmann et al 1987, Villemain et al 1989, Masserini et al 1990, Schattner et al 1996, Cazzullo et al 1998, Printz et al 1999, Sperner-Unterweger et al 1999). One explanation for the deviations between different studies and patient materials may be due to some kind of 'on' and 'off' switches of immune responses during the course of illness. It is also possible that the immunological aberrations are more established among the patients with an advanced form of the disease, while the acute first episode is influenced by a variety of mechanisms affecting the

immunological response. The numbers of total T cells and CD4+ cells have been shown to increase in the course of clinical improvement (Muller et al 1991), and a positive family history of psychiatric diseases seems to correlate with the high number of CD4+ cells and a higher CD4+/CD8+ ratio (Muller et al 1993).

Natural killer (NK) cells, functioning in the vanguard of immune defences against tumors and viral infections, tend to lose a part of their activity in connection with depressive disorders (Caldwell et al 1991, Zisook et al 1994). Some studies have revealed no mean differences in NK cell activity between the schizophrenic or schizoaffective patient groups and their controls (McDaniel et al 1992), but there are also reports of depressed NK cell activity in schizophrenic patients that could be the result of interaction between various factors such as psychotropic medication and physical restraint (Abdeljaber et al 1994). Sasaki et al (1994) found in their follow-up study that after acute exacerbation of schizophrenia, the NK activity was significantly lower on admission than after four and eight weeks from admission. Continuous exposure to phenothiazines may lead to enhancement of NK cytotoxicity. Urch et al (1988) have described reduced NK activity in both depressive and schizophrenic patients, but medical treatment improved the NK activity only in the schizophrenic patient group. In 1999 Sperner-Unterweger et al have detected reduced amounts of NK cells in the acute state of schizophrenic psychosis, and a normalization of the cell number during treatment in first episode patients; in the chronic group the initially low number of NK cells normalized over time.

It seems possible that the alterations in NK cell activity are not specific to schizophrenia, and the endocrinological and noradrenergic aberrations or stress effects may contribute to the above-mentioned abnormalities both in schizophrenia and affective disorders.

High titers of interferons and enhanced production of interferon γ were detected already 15 years ago (Preble and Torrey 1985), but later studies were contradictory (Rimón et al 1985, Rimón and Ahokas 1987). Katila et al (1989) have reported a diminished ability of leukocytes from schizophrenic patients to produce interferon alpha and gamma. Arolt et al (1997) have reported a lowered production of IFN- γ in acutely ill schizophrenic individuals. IL-2 has been the most studied interleukin in the field of schizophrenia research. On the serum level, decreased production of interleukin-2 has been detected (Ganguli et al 1992, Yang et al 1994), and increased serum concentrations of interleukin-2 receptor (Barak et al 1995) and interleukin-6 (vanKammen et al 1999). Levels of CSF interleukin-2 appear to be affected by relapse mechanisms, while peripheral blood levels are not. These changes are specific to interleukin-2, since levels of interleukin-1 alpha seem to be affected by medication withdrawal but not by a change in clinical state (McAllister et al 1995). Circulating levels of TNF alpha have been significantly higher in patients than in controls

(Naudin et al 1996) and it has been hypothesized that TNF alpha and IL-6 reflect the genetic background of disease susceptibility (Naudin et al 1997).

Chemoattractant cytokines, the chemokines, have an important role in the early events of inflammation (Wolpe et al 1988, 1989). They are involved in the inflammatory host responses to foreign pathogens by attracting and stimulating leukocytes. Macrophage inflammatory protein 1 alpha (MIP-1 α) is a chemokine from β -intercrine or C-C superfamily of chemokines which are preferentially chemotactic for monocytes/macrophages. Elevated concentrations of MIP-1 α have been detected in inflammatory CNS diseases (Schall et al 1994, Prieschl et al 1995, Miyagishi et al 1995). Ishizuka et al (1997) have immunohistochemically examined brain tissues from patients with schizophrenia, and have detected glial cells and neurones in the cortex to stain positively for MIP-1 alpha.

Neopterin is a heterobicyclic pteridine compound that is synthesized and excreted by activated macrophages (Huber et al 1984). The activation of the cell-mediated immunity, including viral or bacterial infections, inflammatory reactions and autoimmune diseases increases the neopterin levels in body fluids (Fahey et al 1990, Hagberg et al 1993). Sperner-Unterweger et al (1989, 1992) have reported that, in comparison with healthy subjects, schizophrenic patients show significantly lower urinary levels of neopterin at hospital admission, and that the neopterin concentrations tend to increase during the first days of treatment. Dunbar et al (1992) found no difference between the neopterin concentrations of schizophrenic patients and normal controls, measured both in plasma and urine. The result was the same in the study of Schattner et al (1996), when neopterin was measured from serum samples. There is one report on increased neopterin levels in the serum of acutely ill and recovered schizophrenic patients by Korte et al (1998).

The data on immunological abnormalities in schizophrenia have mainly been based on peripheral blood studies. The few cerebrospinal fluid approaches have concentrated on immunoglobulins (Solomon and Amkraut 1981, Muller and Ackenheil 1995) and cytokines (DeLisi 1996). There are special technical demands affecting especially cytological examination of the cerebrospinal fluid (CSF), e.g. the low total cell count and rapid postpunctional degeneration of the cells (Yam et al 1987), that have made CSF studies difficult to perform. Nevertheless, the requirements for the CSF approach arise from several CNS studies that have emphasized the 'immune privilege' of the brain (Streit et al 1988), and the communicative functions of the blood-brain-CSF barrier (BBB) between the CNS and the periphery (Nathanson and Chun 1989, Yamada et al 1992). The requirements also arise from the fact that in certain neuroimmunological diseases, e.g. multiple sclerosis (Merelli et al

1991) and myasthenia gravis (Müller et al 1990), the changes in the cerebrospinal fluid (CSF) cells are not directly reflected in peripheral blood.

5.1.5. Neuropathology

The first neurohistological study on psychotic patients who could be considered schizophrenics was conducted by Alois Alzheimer in 1887. He described abnormal cortical nerve cells and cortical alterations which were not associated with gliosis. From the first half of the 19th century to the year 1958 cortical atrophy and alterations in basal ganglia and thalamus were reported on several occasions (Falkai and Bogerts 1995). These early neuropathological findings were later regarded as postmortem artefacts (Peters 1967).

The emergence of new brain imaging techniques in the 1980s marked also the start of a new era of neuromorphological studies in the field of schizophrenia research. These recent postmortem studies have particularly pointed to the limbic system as a major locus of pathology in the CNS of schizophrenic patients. The findings have been: reduced volumes or cross-sectional areas of hippocampus and parahippocampal gyrus (Falkai and Bogerts 1986, Brown et al 1986, Jeste and Lohr 1989), reduced cell numbers, cell size or white matter in these areas (Falkai et al 1988, Benes et al 1991a, Heckers et al 1991), and abnormal cell arrangements in hippocampus or entorhinal cortex (Arnold et al 1991). Other findings in limbic brain regions have been left temporal horn enlargement (Bogerts et al 1985, Crow et al 1989) and increased vertical axon numbers and deficits in small interneurons in the cingulate gyrus (Benes et al 1987 and 1991, Benes and Bird 1987). Cortical detections include lower neuronal densities and deficits in small interneurons in the prefrontal cortex and anterior cingulate gyrus (Benes et al 1986 and 1991b), and higher densities of glial and neuronal cells in some prefrontal areas (Selemon et al 1995 and 1998). The studies of basal ganglia, corpus callosum and thalamus have given inconsistent and discrepant findings, and postmortem studies in general have been heavily criticized due to possible artefacts (shrinkage and swelling of brain tissue) and small sample sizes (Bogerts 1993, Falkai and Bogerts 1995, Harrison 1999). One of the most indisputable findings has been the failure to find excessive gliosis in postmortem studies using either immunochemical staining for glial fibrillary acid protein (Roberts et al 1986, Stevens et al 1988, Falkai et al 1999) or Nissl staining (Falkai et al 1988, Pakkenberg 1990).

5.1.6. Neuroimaging

MRI studies have presented general loss of brain tissue (Gur and Pearson 1993, Andreasen et al 1994) and gray matter deficits (Woods and Yurgelun 1991, Zipursky et al 1992, Gur et al 1999) in the brains of schizophrenic patients. The temporal lobe has been the brain parenchymal region with the most consistently documented abnormalities (Turetsky et al 1995, McCarley et al 1999) and especially hippocampus, amygdala and parahippocampal gyrus seem to be affected (McCarley et al 1999). Some researchers have suggested that these changes are nonprogressive and may signify a period of dysregulated brain development in early life (Pfefferbaum and Zipursky 1991, Lim et al 1996). Recently, reports on the correlation between the extent of macroscopic brain alterations and the duration of the psychotic disease have emerged (DeLisi et al 1997, Jacobsen et al 1998, Rapoport et al 1999), thus implicating the contribution of a chronic or remitted progressive pathophysiological process in the CNS of schizophrenic patients.

Functional neuroimaging studies (PET, SPECT, fMRI, MRS, MEG) have produced mostly contradictory or doubtful findings (Weinberger and Berman 1996, Zakzanis and Heinrichs 1999). Most studies have suggested low glucose metabolism in frontal areas, but it seems that hypofrontality, at least in young acute unmedicated schizophrenic patients, is a result of the inability to activate frontal regions during cognition, rather than a baseline decrease in frontal activity. (Parellada et al 1998). Neurochemical brain imaging findings point to elevated striatal D2 receptor density in some patients, unaffected cortical 5-HT_{2A} receptors, and decreased levels of NAA (N-acetyl-aspartate) in the hippocampus and frontal cortex of schizophrenic patients (Soares and Innis 1999). MEG recordings during transitory auditory hallucinations have revealed response delays in schizophrenic patients, suggesting parallel activity on the auditory cortex (Tiihonen et al 1992). Some MEG studies also point to abnormalities of the consecutive preconscious auditory processing in schizophrenia (Pekkonen et al 1999).

5.2. Cerebral blood flow, blood-brain barrier and cerebrospinal fluid

Although the global blood flow of the brain is well autoregulated in the range of 60 to 160 mm Hg arterial blood pressure, the regional cerebral flow fluctuates with regional metabolic activity. Brain blood vessels are very sensitive to Pco₂ but less sensitive to plasma H⁺, because H⁺ cannot get through the BBB. The majority of cerebral capillaries are of the nonfenestrated type and construct an effective barrier against many substances. It is penetrated in only a few areas of the brain. The

BBB is a semipermeable cell layer (the interior wall) of blood vessels in the central nervous system. BBB prevents large molecules, immune cells, and all potentially detrimental substances and foreign organisms (e.g. pathogens) from passing out of the bloodstream and into the CNS (Montemurro and Bruni 1988, Kiernan 1998).

The intermittent capability of solutes, pathogens and cells to cross the BBB indicates an active interaction of endothelium with pathogens and immunocompetent cells before they can penetrate the CNS. The lectin-solute conjugates take an axoplasmic pathway that is comparable but not identical to that followed by viral particles during their retrograde or anterograde transit through the axoplasm. The viruses are transferred to other neurons transsynaptically basically in the same way as the conjugates, but the receptor-mediated transport used by viruses is more specific. Nerves are implicated in both the entry and outlet of antigens into and out of the brain. Antigens generated within the CNS have competence to flight from the brain to lymphoid tissue by passing into the fluid around a cranial nerve and further via the lymph into lymph nodes to start an immune response involving the CNS (Brightman et al 1995).

The CSF is a transparent colourless fluid with a specific gravity ($1.004 - 1.007 \text{ g/cm}^3$) somewhat greater than water. The normal pressure of human CSF as measured in the recumbent position by lumbar puncture varies from 25-70 mm H₂O in infants and from 65-195 mm water in adults.

Under normal conditions it contains scarce protein and has a lower pH, and lower concentrations of glucose, potassium, calcium, bicarbonate and amino acids than blood plasma. However, the sodium, chloride, and magnesium content is greater in the CSF. A small number of cells is usually present in the CSF: 0-8 cells/ mm³ in infants and 0-5 cells/mm³ in adults is considered normal (Davson and Segal 1996).

The total volume of CSF within the ventricular system and the subarachnoid space is estimated to be 80-150 ml and the ventricular system alone is assumed to contain from 15-40 ml of CSF, and 75 ml surrounds the spinal cord. Under standard conditions, CSF is formed by the choroid plexus present within the four cerebral ventricles, the choroid plexus of the lateral ventricles producing the most. The formation rate is approximately 0.35 ml/min or 500 ml/day, and it replaces the total volume of CSF about 2-3 times over in 24 hours (Nolte 1999). In humans, circadian variation in CSF production has been demonstrated, involving a nocturnal increase in production that reaches twice daytime values (Nilsson et al 1992). Especially under pathological conditions, a considerable amount of CSF may also be produced at sites other than the choroid plexus; the ependymal lining of the ventricles and the endothelium of brain capillaries have been considered potential sites of

extrachoroidal CSF production. As much as 12-20% of the total CSF volume may be extracellular fluid of capillary origin (Nolte 1999).

The circulation of cerebrospinal fluid proceeds from the lateral ventricles into the third ventricle through the interventricular foramen. A minor reflux of CSF into the contralateral lateral ventricle is believed to occur. From the third ventricle, the CSF arrives at the fourth ventricle via the narrow cerebral aqueduct, and CSF leaves the ventricular system at the level of the medulla oblongata through three apertures: the midline foramen (of Magendie) and the paired lateral foramina (of Lushka). These apertures open into enlargements of the subarachnoid space known as the cisterna magna and the cisterna pontis, respectively (Montemurro and Bruni 1988, Kiernan 1998). The circulation of CSF within the subarachnoid space also follows an arranged course: from the lateral foramina of Lushka and the cisterna pontis, the CSF flows anteriorly along the base of the brain and ascends slowly along the Sylvian fissure and the lateral convex and medial surfaces of the hemispheres. From the midline foramen of Magendie and cisterna magna, the CSF flows forward over the cerebellar hemispheres toward the tentorial incisure and also downward into the subarachnoid space surrounding the spinal cord. The downward flow of CSF around the spinal cord in normal humans has been a subject of debate. There are suggestions that no true spinal flow occurs other than that afforded by gravity, but also demonstrations of a rapid descent of spinal CSF that contrasts with a slower endocranial circulation. The significance of a normal downward spinal flow of CSF is that it provides a substitute path for CSF absorption under certain pathological conditions, and it also forms the basis for diagnostic CSF sampling from the lumbar cistern. Only a small amount of CSF is thought to reach the fourth ventricle by ascending the central canal of the spinal cord. In fact, the central canal of the spinal cord is occluded in most adults after the age of 20 (Davson and Segal 1996, Montemurro and Bruni 1988, Kiernan 1998).

The most important course by which CSF enters the bloodstream is through the arachnoid villi. These are microscopic projections of pia-arachnoid mater that extend into venous channels providing CSF-vascular interfaces; the villi are finger-like projections consisting of a cellular and fibrous connective tissue core surrounding fluid-filled spaces that are continuous with the subarachnoid space. Villi function as one-way valves returning CSF from the subarachnoid space to the dural venous sinuses, as the hydrostatic pressure of the CSF usually exceeds venous pressure. The precise nature of transport between the subarachnoid space, villus spaces and the venous channels is still disputed. Different pathways of CSF absorption have also been described. Sparse amounts of CSF are known to be absorbed via pial vessels and across the walls of cerebral capillaries within the brain parenchyma. Some absorption also occurs via lymphatic channels

bordering extensions of the subarachnoid space that surround cranial and spinal nerves. The presence of arachnoid villi and granulations around spinal nerve roots and their proximity to veins is consistent with the absorption of CSF at spinal cord levels. The involvement of these alternative routes of absorption is thought to be particularly important under pathological conditions such as hydrocephalus (Davson and Segal 1996).

The brain and spinal cord are rendered floatable by the CSF medium in which they are suspended. This supports and protects the nervous system against rapid movements and trauma. The CSF is considered to be nutritive for both neurons and glial cells, and the CSF provides a vehicle for removing waste products of cellular metabolism from the nervous system. It thus functions like a lymphatic system. The CSF also preserves the consistency of the ionic composition of the local microenvironment of the cells of the nervous system. The extracellular space of the brain freely interconnects with the CSF compartment and therefore the composition of the two fluid compartments is similar. The existence of a number of biologically active compounds (metabolites, neurotransmitters, hormones, releasing factors) within the CSF suggests that it may function as a transport system. Since the CSF and brain extracellular space are connected, analysis of the composition of the CSF provides diagnostic information about the normal and pathological states of the CNS function (Kiernan 1998, Nolte 1999).

5.3. Cerebrospinal fluid cells

The upper limit of the normal total cell count in the CSF of adult individuals varies from 2500 to 5000 cells $\times 10^3/l$ in specimens taken from the lumbar area (Oehmichen 1976), depending on the method used. Normal CSF contains mononuclear cells, and the granulocytes and red blood cells are occasional and rare findings. In the normal differential count, two thirds of the CSF mononuclear cells are lymphoid cells and the remaining one third consists of cells from the mononuclear phagocyte lineage (monocytoids and macrophages) (Cook and Brooks 1980, Taskinen 1983). The distribution of CSF mononuclear cells is age-dependent: newborn infants exhibit a reverse differential count with one third lymphocytes and two thirds monocytoids/macrophages; this gradually turns into a normal adult-type distribution during childhood and adolescence (Oehmichen 1976, Cook and Brooks 1980).

The increase in the total number of CSF cells, pleocytosis, is most often associated with the inflammatory diseases of the CNS (acute bacterial or viral infections), and in some instances with intracerebral haemorrhages and CNS neoplasms (Taskinen 1983). A normal cell count does not, however, exclude CNS disease. Pathologic differential counts without pleocytosis have been detected e.g. in multiple sclerosis, some forms of epilepsy and neoplasms, as well as in the recovery phase of CNS injuries or infections (Cook and Brooks 1980). Changes in the proportions of CSF lymphoid cells and mononuclear phagocytes/macrophages may also reflect the activity of the chronic inflammatory or degenerative diseases.

5.3.1. CSF lymphocytes

The main lymphocyte population of normal CSF consists of small resting lymphocytes, while the appearance of enlarged, basophilic or activated lymphocytes or plasma cells is usually associated with CNS immunoactivation (Taskinen 1983). In the CSF the T/B lymphocyte ratio tends to be higher than in peripheral blood (Oehmichen 1976). Changes in the proportions of T-lymphocyte subsets, CD4+(helper/inducer) and/or CD8+ (cytotoxic/ suppressor) cells and in the CD4+/CD8+ ratio in the CSF have been reported to occur in various central nervous system diseases of inflammatory or autoimmune origin (Pirttilä et al 1987, Kölmel and Sudau 1988, Rotteveel and Lucas 1990). Both upward and downward shifts of CD4/CD8 ratios can be signs of immunological abnormalities. The ratios have mainly been elevated in most neuroimmunological diseases, whereas in HIV infection a decreased CD4/CD8 ratio can be observed already at an early stage of the illness (Müller et al 1993).

5.3.2. CSF mononuclear phagocytes and macrophages

The cells of mononuclear phagocyte / macrophage lineage detected in the CSF have two main tasks: the removal of foreign material (scavenger function) and the interactions with lymphoid cells (immunologic function). Experimental models of nerve injury have exposed the critical role of macrophages in both degenerative and regenerative actions in the nervous system (DeGroot et al 1992). In certain neurological diseases, the action of resident macrophages seems to play a role in the disappearance of neurons (Streit et al 1988).

Morphologically, immunophenotypically and functionally, the cells of the monocyte/macrophage lineage are related to microglia (Streit et al 1988, DeGroot et al 1992, Kettenmann et al 1993).

Microglia form a regularly spaced network of resident glial cells throughout the central nervous system. Microglial cells appear to have an essential role in the interaction between CNS and the immune system. This interplay is mediated by neuropeptides, cytokines and other soluble mediators of intercellular communication (Zielasek and Hartung 1996). Microglial activation tends to occur at an early stage of CNS response to injury, so that it often precedes the reactions of any other cell type in the brain. This transformation of microglia from a resting to an activated state occurs in a relatively stereotypic pattern displaying proliferation, migration, and changes in morphology, immunophenotype and function. The first stage of activation is non-phagocytic, and in the second stage the activated microglia transform into phagocytic cells, also known as microglia-derived brain macrophages. In addition, microglia have a strong antigen-presenting function and a pronounced cytotoxic function (Kreutzberg 1996). The cytotoxic vs. protective functions of microglia are modulated by cytokines and neurotransmitters. IFN- γ activates both macrophages (resulting in e.g. neopterin production in inflammatory responses, Huber et al 1984) and microglia, but other cytokines, such as TGF- β 1 or IL-4, downregulate microglial and macrophage cytotoxicity (Loughlin et al 1993).

Macrophages and microglia have also been suggested to have neurodevelopmental actions. Activated microglial cells have been thought to play several important roles in brain tissue development, associated with programmed cell death. (Streit et al 1988, Kettenmann et al 1993, Upender and Naegele 1999).

6. PURPOSE OF THE STUDY

The objective was to investigate possible cytological aberrations within the central nervous system of schizophrenic patients by analyzing the levels of the main immunocompetent cell categories and their inflammatory products from samples of cerebrospinal fluid. To elucidate the effect of psychiatric treatment on these parameters, a follow-up section was included in these studies.

The specific aims were:

1. To investigate the cerebrospinal fluid cell numbers and distributions in schizophrenic patients during an acute psychotic episode.
2. To perform a morphological analysis of mononuclear cells from schizophrenic patients.
3. To clarify T lymphocyte subset proportions in the cerebrospinal fluid of schizophrenic patients and to compare them in corresponding peripheral blood analysis.
4. To determine the cerebrospinal fluid concentrations of the inflammatory products neopterin and MIP-1 α in schizophrenia.

7. MATERIAL AND METHODS

7.1. Patients

The study protocol was described to the subjects, and their written informed consent was obtained. The Ethics Committees of Hesperia Hospital and of Helsinki University Hospital gave their approval.

From a series of 63 acutely psychotic patients admitted voluntarily or involuntarily to Hesperia Hospital (Department of Psychiatry, Helsinki City Hospital) during the years 1991-1993, 50 individuals (28 women, 22 men) were included in these studies. The DSM-III-R diagnostic criteria (American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, Revised Third Edition. American Psychiatric Association, Washington, DC, 1987) were applied to confirm that the psychosis of the patients belonged to the schizophrenia spectrum (schizophrenia, schizophreniform disorder, schizoaffective disorder). Patients with other diagnoses were ruled out, as well as subjects with serious physical diseases, alcoholism, or drug abuse. Acute infections were excluded clinically and by routine laboratory infection markers in peripheral blood and in CSF. The CSF/serum ratio for albumin was 4.2 ± 1.5 (mean \pm SD) in the patient group, excluding significant blood-brain-barrier damage of the patients.

The mean age of the total patient population was 31.9 years (SD 8.2, range 18-53). Twenty-nine patients were on their first admission and they had not been on neuroleptic medication before. The remaining 21 patients had been previously treated for at least one psychotic episode (3.2 ± 4.5 episodes including the current one, range 1-26), but they had been drug-free for at least three months prior to admission. The symptoms of the patients were rated with the 18-item Brief Psychiatric Rating Scale (BPRS) (Bech et al 1986), rating each item from 0 to 6.

The demographic and clinical characteristics of the patients in each individual study are given in Tables 1 and 2.

Table 1. Demographic characteristics of the patients in Studies I-IV

	N	Female/male	Age (means \pm SD, range)
I	31	15/16	31.2 \pm 7.6, 20-54
II	35	16/19	33.3 \pm 8.7, 18-53
III	30	17/13	30.9 \pm 8.8, 18-43
IVa	11	6/5	31.2 \pm 8.8, 18-43
IVb	8	3/5	34.4 \pm 9.6, 18-43

Table 2. Clinical characteristics of the patients in Studies I-IV

	FA/RE ^a	BPRS18 ^b	HD ^c	CPZeq ^d
I	18/13	59.4 \pm 11.2	4.1 \pm 2.3	426 \pm 288
II	20/15	56.0 \pm 14.1	4.4 \pm 2.3	377 \pm 215
III	17/13	57.5 \pm 12.6	4.4 \pm 2.4	369 \pm 230
IVa	6/5	60.3 \pm 11.2	4.5 \pm 1.8	390 \pm 244
IVb	4/4	58.4 \pm 10.6	4.3 \pm 1.7	400 \pm 288

^a first admission / re-entry patients,

^b BPRS scores (means \pm SD),

^c days in hospital before sample collection (means \pm SD)

^d neuroleptic medication dosage per day in chlorpromazine equivalents (means \pm SD)

7.2. Reference populations

The control group in Studies I-III consisted of patients examined at the Outpatient Department of Neurology at the Helsinki University Central Hospital for the following symptoms: headache, leg or

arm pain and/or paresthesia, vertigo, facial pain, vasovagal attack, hypacusis, and tinnitus. One of the control subjects had a brain infarction of the right hemisphere nearly two years after CSF sampling. In the rest of the controls no evidence of inflammatory or CNS disease emerged at examination during a follow-up period of 2.5 to 6 years. In Study I the control population consisted of 21 individuals, 6 men and 15 women. The mean age of the reference series was 43 (range 27-64) years. The control population in Studies II and III consisted of 46 outpatients; the female/male ratio was 25/21. The mean age of this reference series was 33 (range 18-65) years.

In Study IV the controls were unmedicated, normotensive paid volunteers from the hospital staff. Subjects with a family history of psychiatric illness in first-degree relatives were excluded. Psychiatric diagnoses of control subjects were ruled out by the Structured Clinical Interview for DSM-III (SCID). The control population participating in the neopterin study consisted of 10 persons (3 women and 7 men), aged 34, range 20-57 years, and the MIP-1 α study was conducted with a control group that comprised of three women and five men, aged 31 (range 20-57) years.

Demographic characteristics of reference populations as regards the individual studies are reported in Table 3.

Table 3. Demographic characteristics of control and reference populations in studies I-IV

	N	Female/male	Age (means \pm SD, range)
I	21	15/6	43.0 \pm 9.8, 27-64
II and III	46	25/21	32.7 \pm 9.8, 18-65
IVa	10	3/7	33.6 \pm 12.8, 20-57
IVb	8	3/5	31.3 \pm 11.7, 20-57

7.3. Collection and preparation of specimens

The collection of CSF and PB samples from the schizophrenic patients was performed on day 4.6 \pm 2.4, range 0-7 days after admission. During this period the patients were medicated with a mean

neuroleptic dose of 396 ± 242 mg chlorpromazine equivalents. The principal antipsychotics in use were chlorpromazine and haloperidol. Two patients used additional benzodiazepines and one was on lithium. No other drugs were administered.

After overnight fasting and 30 minutes' bed rest, blood was drawn at 8-9 o'clock a.m., and immediately after that lumbar punctures were performed in lateral decubitus position. The CSF was collected in fractionated aliquots in chilled tubes. For the quantitative mononuclear cell analysis 1 ml of native CSF was fixed in 1 ml of 96% ethanol and the specimens were filtered through Millipore filter (Millipore Corp., Bedford, MA, USA) and stained according to the Papanicolaou method. Cytocentrifuged smears were prepared from samples of 4 ml of unconcentrated CSF in cell culture medium that was kept on ice and centrifuged ((Shandon Cytospin; 800rpm/8-10min) within 30 minutes in order to prevent postpunctional cell degeneration. The native unconcentrated CSF samples for neopterin and MIP-1 α measurements were immediately frozen at -70°C until assayed.

Blood samples were taken from an antecubital vein and promptly centrifuged at 4°C at 3000 rpm for 10 minutes. The separated serum was immediately frozen at -70°C until assayed. Blood mononuclear cells were separated from heparinized PB samples by Ficoll-Hypaque density gradient centrifugation, and cell smears were processed by cytocentrifugation as described above.

Cytocentrifuged CSF samples were stained with May-Grünwald-Giemsa (MGG) and analyzed in order to observe detailed morphological features of the cells and to exclude samples with red blood cell contamination; samples with >20 red blood cells / high power (40x) field were rejected.

7.4. Evaluation of total cell count and the differential count of mononuclear cells

The Millipore filtration CSF slides were prepared by commercial Millipore filtration equipment using cellulose acetate filters. The filtration was assisted with gentle negative pressure. The slides were stained by the Papanicolaou method, and were examined by light microscopy at 40 x magnification for quantitative determinations, and at 100 x magnification for counting the main mononuclear cell distributions. The cells per visual field were counted and the number of total visual fields were evaluated to determine the absolute cell numbers of each CSF sample. The differential counts were performed by analyzing at least 100 cells from each slide on a morphological basis. The method has been described in detail by Kölmel (1977) and Taskinen (1983).

7.5. Detailed morphological analysis of mononuclear cells

The morphological details of CSF and PB cells were examined by light microscopy on cytocentrifuged samples stained by MGG. The objective magnification of 100 x was used, and at least 60 cells per patient sample were analyzed. On a morphological basis the cells were divided into mononuclear phagocytes/macrophages and lymphocytes. The lymphocytes were further categorized as normal or activated, on the basis of their nuclear and cytoplasmic contour and staining properties. The calibration of microscopic analysis was performed by blind comparison between two analysts.

7.6. Phenotyping of lymphocytes

Phenotyping of CSF and PB lymphocytes to CD4+ and CD8+ T cells was carried out with the 3-layer indirect immunoperoxidase technique on air-dried cytocentrifuge smears. The following primary monoclonal mouse antihuman antibodies were used: leu 3a+3b (Becton Dickinson, Mountain View, Ca, USA) for detecting CD4+ lymphocytes and OKT-8 (Ortho Diagnostic Systems Inc., Raritan NJ, USA) for detecting CD8+ cells.

The stained specimens were analyzed by light microscopy at 40x and 100x magnification. From CSF cell preparations, 68+/-5 lymphocytes were analyzed for each surface marker. As for PB samples, a total of 100 lymphocytes were counted for each monoclonal antibody.

7.7. Neopterin assay

The HENNINGtest[®] Neopterin –radioimmunoassay was applied to measure the neopterin levels in the CSF and serum samples. This RIA method employs the ¹²⁵I-Neopterin tracer. Samples and neopterin standards were mixed with tracer and precipitating antiserum, then incubated for one hour at room temperature under the exclusion of light. After washing and pelleting by centrifugation, the radioactivity was measured by a gammacounter. The mean count rates of double samples were related to the count rate of zero standard and the corresponding neopterin concentrations in nmol/l were read from the standard curve.

7.8. ELISA for MIP-1 α

A quantitative sandwich enzyme immunoassay technique (Quantikine MIP-1 α ; R&D Systems) was used to determine the MIP-1 α concentrations from CSF samples. A monoclonal antibody specific for MIP-1 α was precoated onto a microplate. According to the manufacturer's instructions, standards and samples were pipetted into the wells in duplicate, and any MIP-1 α present was bound by the immobilized antibody. Unbound substances were removed by washing, and an enzyme-linked polyclonal antibody specific for MIP-1 α was added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution was added to the wells, and color developed in proportion to the amount of MIP-1 α bound in the initial step. The color development was stopped by a specific stop solution, and the intensity of the color was measured by a microplate reader at 450 nm.

7.9. Statistical methods

Descriptive statistics are given as means \pm standard deviations (SD) with the exception of Study I, in which means \pm SEM (standard error of means) were used and the reference values for T lymphocyte subsets were obtained from the examinations of the control subjects with the percentile method (Herrera 1958), which estimates the 95% normal range.

The Mann-Whitney U test, rank transforms and ANOVA were used to study the between group values and Wilcoxon matched pairs test was the statistical method applied to compare the patients' CSF cytology before and after treatment. The correlations were statistically analyzed by the Spearman rank order correlation test.

8. RESULTS

8.1. T lymphocyte subsets

Comparison of the percentages of CSF lymphocyte subtypes (Study I) separately (CD4+ and CD8+ cells) or the CD4/CD8 ratios between the whole group of schizophrenic patients (N=31) and healthy controls (N=21), brought forth no statistically significant differences (Table 4). The schizophrenic patient group exhibited obviously wider ranges (16-73% for CD4+ and 15-52% for CD8) than did the reference population (27-68% for CD4+ and 21-46% for CD8+). As first-admission and re-entry patients were analyzed separately, no statistical difference could be detected between the controls and first-timers (N=18), but in the re-entry group (N=13) the range was more narrow regarding both CD4+ and CD8+ percentages, and there was a statistically significant ($p=0.022$) tendency towards lower CD8+ lymphocyte percentages in the CSF (Table 5).

Determination of the 95% reference limits of the control population, and consideration of the proportions of both lymphocyte subsets together, revealed the aberrant CD4+ and/or CD8+ distributions in the majority of the patients (23/31; 74%). Remaining below the reference limit was a more prevalent finding concerning both lymphocyte subtypes. As many as 15/18 (83%) of the first-admission patients were outside the reference limits, while in the re-entry group 8/13 (62%) of the patients had CD4+ and/or CD8+ percentages outside the normal range. All the patients with high CD8+ percentages were from the first-admission group (Figure 1).

There was a negative correlation between the total count of CSF cells and the proportion of CD8+ lymphocytes ($r=0.61$), and between the patients' age and CD4+% ($r=0.76$) or CD4/CD8 ratio ($r=0.67$) in the CSF. Female and male patients did not differ in their lymphocyte subtype presentation in the CSF (*unpublished data*).

The patients screened for both CSF and PB cells (N=15) expressed alterations in the distribution of T cell subsets in the CSF, while the T cell subsets in PB were within the reference limits. The

CD4/CD8 ratios in the CSF were both high and low, while the deviations seen in the PB (5/15) were above the upper reference limit. No correlative association could be detected between CSF and peripheral blood lymphocyte subset findings of the patients. The correlation coefficients were – 0.21 for CD4+%, 0.11 for CD8+% and 0.05 for CD4/CD8 ratio.

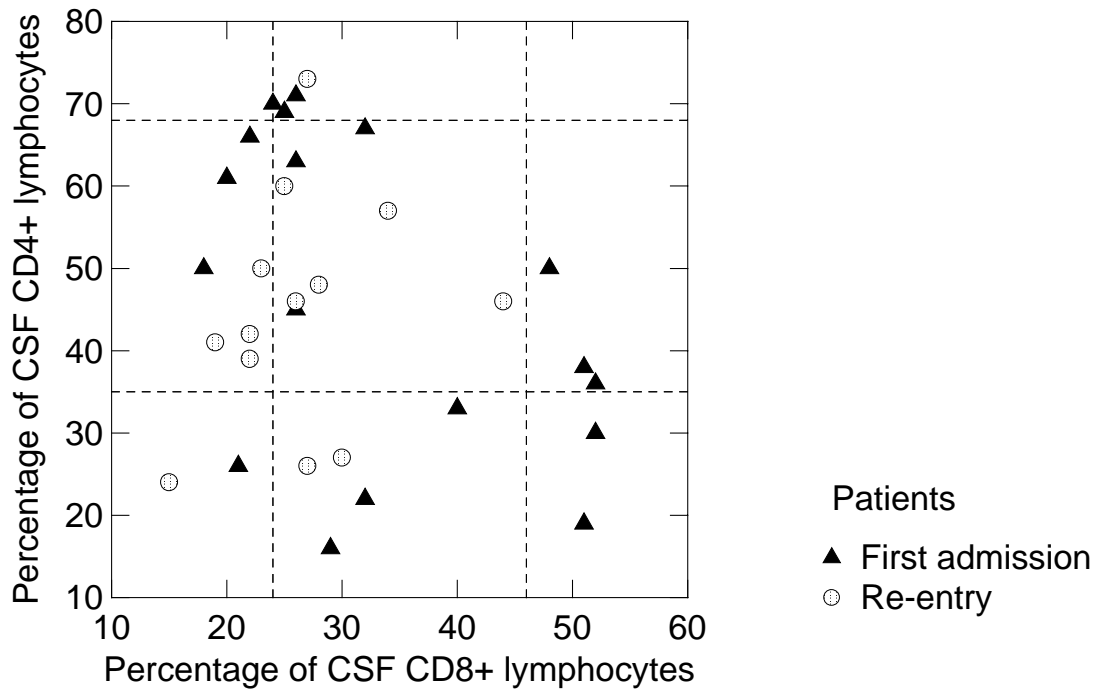


Figure 1. Percentages of CD4+ and CD8+ lymphocytes in the CSF of first admission and re-entry patients with acute schizophrenia. Dashed lines show the lower and upper 95 percentile reference limits of the control subjects.

8.2. Mononuclear cell distribution

The microscopic examination of the CSF Millipore filtration slides from 35 psychotic patients and 46 control individuals (Study II) revealed highly significantly (F -ratio=47.34, $df=1, 16$, $p<0.0001$) elevated proportion of cells morphologically classified as mononuclear phagocytes / macrophages (Figure 2).

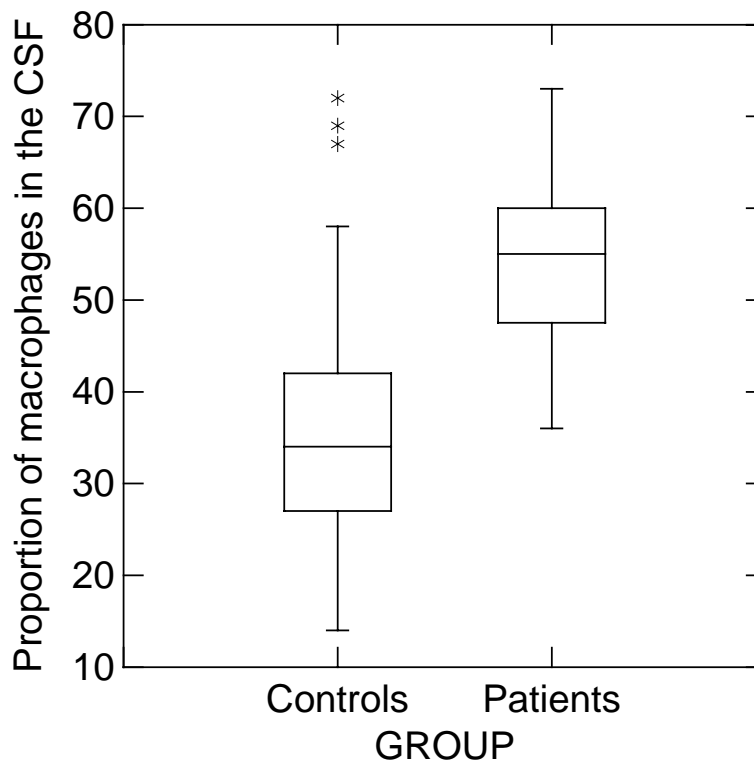


Figure 2. The distribution of mononuclear phagocytes / macrophages (MP %) in the cerebrospinal fluid of schizophrenic patients and normal controls.

No statistically significant difference was detected in the total CSF cell counts of patients and controls (Table 4). The distribution of mononuclear cells was so deviant, however, that in addition to the proportional difference of mononuclear cells, also the absolute numbers of monocytes/macrophages were significantly ($p < 0.001$) elevated in the schizophrenic patient group in spite of the normal total cell count in the CSF (*unpublished data*). A significant tendency ($p < 0.05$) towards a higher CSF monocyte / lymphocyte ratio was observed in the re-entry patient group ($N=15$) as compared with first-admission patients ($N=20$) (*unpublished data*). No statistical differences in the appearance of the mononuclear cells in the CSF could be found with regard to the sex of the patients. The relative and absolute values for mononuclear cells did not correlate significantly to the patients' age, BPRS scores or medication (the duration of medical treatment prior to sample collection, and the dose of medication in chlorpromazine equivalents).

A statistically significant tendency towards normalization of the cytological picture (i.e. decrease in the elevated frequency of monocytes/macrophages) was observed in the CSF of those psychotic

patients who took part in the follow-up section of the study ($N=13$, $df=1$, 18 , $P<0.05$) after a few weeks' treatment with typical neuroleptics. The absolute numbers of monocytes in the CSF of the patients did not change significantly in the course of the treatment.

8.3. Morphological analysis of mononuclear cells

Analyzed from the MGG stained cytocentrifuge slides (Study III), the cytological profiles of the CSF samples from schizophrenic patients ($N=30$) at the initial phase of hospital treatment differed clearly from those in the CSF of healthy controls ($N=46$). The detected dissimilarities concerned both the differential counts of mononuclear cells (as in Study II obtained with a different methodology) and the morphological details of the lymphoid cells.

The most outstanding lymphocyte finding was an accumulation of phenotypically deviant cells with morphological characteristics of activation (Figure 3). The size of these cells ranged from small to medium and large, and the morphological attributes consisted of basophilic cytoplasm, convoluted nuclei and an irregular nuclear membrane, prominent nucleoli and dispersed chromatin. All in all, the morphological features of these cells are typical for stimulated or immunoactivated lymphocytes, although the majority of these cells in the CSF slides of schizophrenic patients did not fulfill the size criteria of stimulated lymphocytes. The dominating cell population in the CSF slides of control individuals represented small, resting lymphocytes; large stimulated lymphocytes were less frequent, and small to medium-sized lymphocytes with morphological signs of activation were virtually absent. Pooled together, the proportion of lymphocytes with characteristics of stimulation or activation (including both "normal" large stimulated cells and "atypical" small to medium-sized lymphocytes) exceeded the cohort of normal resting lymphocytes in the schizophrenic patient group. Compared to the controls, the difference was statistically highly significant ($p<0.001$, Mann-Whitney U test) (Table 4).

No correlations of lymphocyte activation stage were detected with the patients' age, BPRS scores, the absolute number of cells in the CSF, or medicational status (treatment days before sample collection, and the neuroleptic dose). Female and male patients did not differ from each other as a group in their expression of lymphocytes with morphological activation signs.

Analysis of the follow-up samples from the patients after 3-4 weeks of hospital treatment with conventional neuroleptics did not point to significant changes in the lymphocyte profiles during this

period, whereas the macrophage/lymphocyte distribution exhibited a tendency towards normal appearance ($p < 0.05$, Wilcoxon matched pairs test). The last-mentioned finding was in line with the detection of normalization in the CSF cell arrangement during the course of treatment, as observed from Millipore filtration slides in Study II.

The monocytoïd cells of schizophrenic patients represented pleomorphic maturational stages on the basis of the cytological details (Figure 3). These cells ranged from juvenile mononuclear phagocytes with a compact kidney shaped nucleus and homogeneous cytoplasm including some small acidophilic granules to mature macrophages with a lobulated irregular nuclear shape and a voluminous cytoplasm with numerous vacuoles. Overt lipophages with larger cytoplasmic vacuoles were rarely seen.

In the schizophrenic patient group, occasionally “rosettes” i.e. aggregates between lymphocytes and macrophages were encountered in the CSF slides. Sporadic findings of polymorphonuclear leukocytes comprised less than 1% of the cell populations, both in schizophrenic patients and in normal controls.

8.4. Levels of Mip-1 α and Neopterin

Measuring the concentrations of CSF MIP-1 α did not reveal statistical differences between the control and patient populations, and the patients’ MIP-1 α concentrations did not change during the course of medical treatment. The concentrations in the group of 8 control individuals were 5.1 ± 0.7 pg/mL (mean \pm sd). In the patient group (N=8) the corresponding values were 4.7 ± 0.3 pg/mL at admission, and 4.7 ± 0.4 pg/mL after the treatment period.

The CSF neopterin concentrations of 10 control subjects were 2.9 ± 1.5 nmol/l (mean \pm sd) and those of 11 schizophrenic patients beginning their hospital treatment were 2.8 ± 2.2 nmol/l. There was no statistical difference between the patient and control groups. The follow-up samples of these 11 schizophrenic patients expressed concentrations 2.7 ± 2.1 nmol/l, which did not reach statistical difference to the neopterin concentrations from either the control population or from the same patients at admission.

Table 4. Main results of Studies I-IV concerning the CSF findings of schizophrenic patients and normal controls (means \pm SD).

	Patients	Controls	p-values
I	N=31	N=21	
CD4+ (%)	46 \pm 3 ^a	52 \pm 2 ^a	n.s.
CD8+ (%)	30 \pm 2 ^a	33 \pm 2 ^a	n.s.
CD4/CD8	1.71 \pm 0.15 ^a	1.63 \pm 0.08 ^a	n.s.
II	N=35	N=46	
Cell count	1466 \pm 1070	1359 \pm 755	n.s.
Monocytes	53.9 \pm 9.1	35.7 \pm 13.5	<0.001
Lymphocytes	46.3 \pm 9.1	63.9 \pm 13.8	<0.001
III	N=30	N=46	
Resting Ly ^b	45 \pm 13	81 \pm 8	<0.001
Activated Ly ^b	55 \pm 13	19 \pm 8	<0.001
IVa	N=11	N=10	
Neopterin ^c	2.8 \pm 2.2	2.9 \pm 1.5	n.s.
IVb	N=8	N=8	
MIP-1 α ^c	4.7 \pm 0.3	5.1 \pm 0.7	n.s.

^a means \pm SEM^b Ly=lymphocytes (%)^c concentration (Nmol/l for neopterin, pg/ml for MIP-1 α)**Table 5.** Main results of Studies I-IV concerning the statistically significant CSF findings of first admission (FA) and re-entry (RE) patients analyzed separately (percentages, means \pm SD)

	FA	RE	Difference: FA/RE	Difference to controls
CD8+	33 \pm 3 ^a	26 \pm 2 ^a	n.s.	<0.05 (RE)
Monocytes	49.8 \pm 8	58.2 \pm 8.3	<0.05	<0.001 (FA&RE)
Lymphocytes	50.5 \pm 7.7	41.8 \pm 8.3	<0.05	<0.001 (FA&RE)

^a means \pm SEM

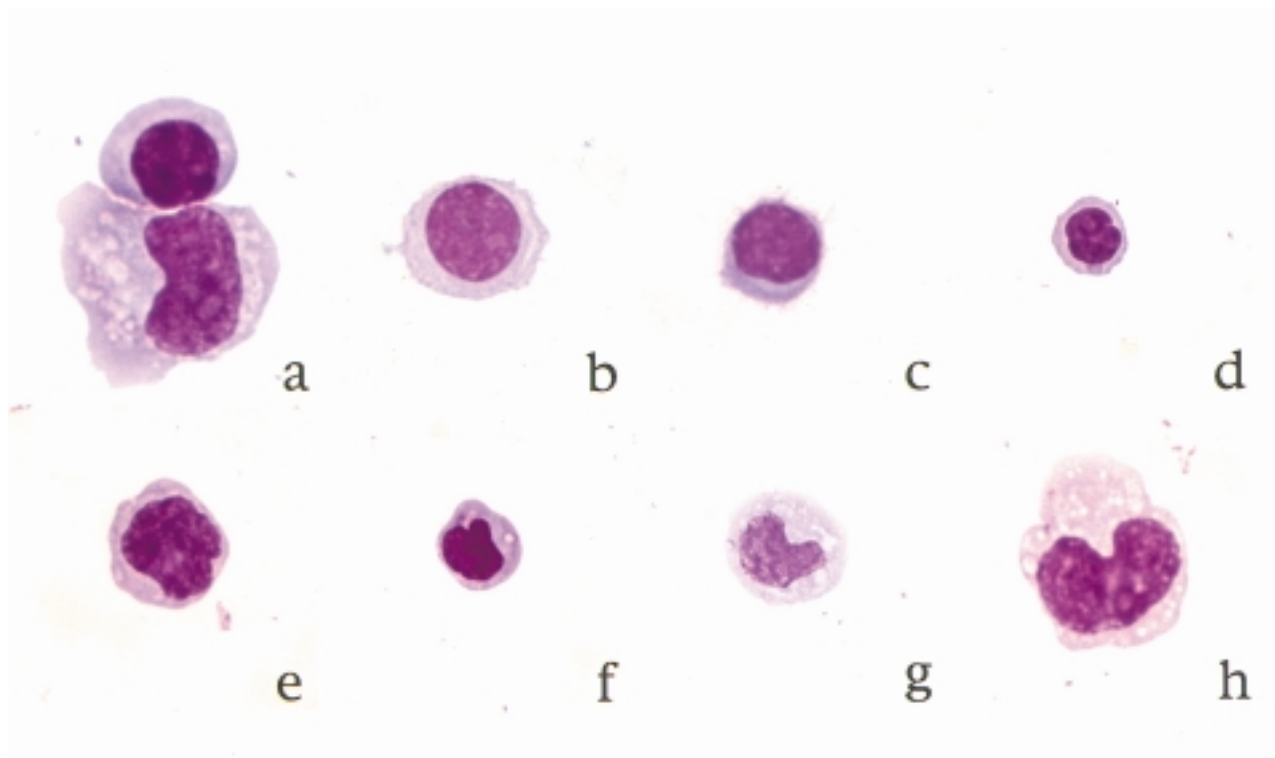


Figure 3. Morphological features of mononuclear cells in the CSF from schizophrenic patients. Cytocentrifuged slides were stained with May-Grünwald-Giemsa stain. a) Adhesion of an activated lymphocyte (above) to a mature macrophage with vacuolated cytoplasm. b) Large activated lymphocyte with dispersed chromatin and conspicuous nucleoli c) Medium-sized activated lymphoplasmocytic cell with a basophilic cytoplasm and perinuclear halo. d) Small-sized activated lymphocyte with a convoluted nucleus and prominent nucleoli. e) Large lymphoblast with a cerebriform, convoluted nucleus. f) Monocytic cell/immature macrophage with a folded vermicular nucleus and a cytoplasmic vacuole. g) Macrophage with a kidney shaped nucleus and a pale voluminous cytoplasm. h) Mature large macrophage.

9. DISCUSSION

9.1. Methodological limitations

In relation to the fact that the patient materials of CSF studies on neuropsychiatric disorders have generally been heterogeneous and few in number, the quantitative and qualitative characteristics of the patient and control populations can be regarded as representative in Studies I, II, and III, and moderate in Study IV. Both first-admission and re-entry patients were included, and the total in each subgroup was sufficient for statistical comparisons between the groups in the first three studies. Calculations with correlation coefficients did not point to any significant variations in the CSF findings, as regards the patients' age, duration of illness, number of treatment episodes, medication or clinical status, but in this respect the number of patients can be considered too limited for drawing reliable conclusions on the role of these factors. The drug-free period of at least three months prior to hospital admission was an advantage of the patient materials, and it lessens the possibility of medication effect on the findings of these studies. Unfortunately, medication effect cannot be ruled out entirely, since the CSF samples could not be obtained before starting the medical treatment. The treatment period was, however, quite short and the follow-up part of the studies did not bring out significant changes in cell or cytokine parameters in the course of drug treatment. An exception was the normalization tendency in the mononuclear cell distribution; this finding contradicts the expectation of drug-induced immunological aberrations. Furthermore, previous cytological studies have suggested that short-term medical treatment with conventional neuroleptics does not significantly enhance the pathological features in lymphocyte populations. A correlation between the neuroleptic dose and the number of CSF cells has been detected in one study, but the variation was within normal range, and there was no relationship between the cell frequency and the duration of medical treatment (Wahlbeck et al 2000).

The reference populations in Studies I, II and III consist of neurological outpatients with mild symptoms and no evidence of CNS disease. In spite of the fact that these individuals were not randomly selected healthy subjects, they are to be considered as normal controls due to the thorough and longitudinal exclusion of any disease process. A possible weakness of these reference groups is that psychiatric disorders were ruled out by mere clinical estimation and not with a structured clinical interview, which was the case in the normal control group in Study IV.

The cytological methods of CSF examinations should be selected on the basis of two requirements: high cellular yield and minimal cellular artefacts (Kölmel 1976, Taskinen 1983). The application of flow cytometry (FACS), the method that enables double-staining, was excluded at the pilot phase of the study, as the total cell counts of the samples were found to be normal or low. The sensitivity of FACS in the evaluation of diagnostic CSF cytology is relatively low (Cibas 1995), and the methods derived from immunocytochemistry have been regarded as more reliable for the examination of surface markers on CSF cells, particularly for samples without pleocytosis (Windhagen et al 1999). The combination of Millipore filtration and cytocentrifugation is considered to be the optimal technique for the investigation of CSF mononuclear cells, due to high cellular harvest with the filtration method and well preserved cytomorphology with cytocentrifugation (Gondos and King 1976, Taskinen 1983, Kobayashi et al 1992). These methods permit the reliable quantification of CSF cells and the differential count and morphological analysis of mononuclear cells in the CSF, as well as division into the main lymphocyte subtypes by application of immunoperoxidase staining. The signs of activation nevertheless remain to be evaluated indirectly on a mere morphological basis in the absence of double-staining activation markers, which require the use of flow cytometry. Therefore, it should be borne in mind that the expression 'activated' used in these studies is associated with the morphological features of the CSF cells, and the activated status of the cells is not verified by activation markers.

9.2. The subsets of T lymphocytes in the CSF and PB

The results of the T lymphocyte surface antigen study (**Study I**) point to alterations of the immunocompetent cell composition in the cerebrospinal fluid of schizophrenic patients in the acute phase of the disease. Although the findings concerning CD4 positive and CD8 positive cells and CD4/CD8 ratios varied widely in the patient group, thus covering the statistical significance of deviations at group level, the analysis of the proportions of these cell subsets together revealed that 74% of the patients had an abnormal CD4 and/or CD8 composition in their CSF. As both high and low CD4/CD8 ratios have been detected in a variety of CNS diseases affecting the immune system, the wide-range finding can be explained by three mechanisms: the heterogeneity of the disease itself, the variety of factors affecting the immunological response in the acute phase of the psychotic episode, and the changes in immune reactions during the course of the illness. In this study the finding of a statistically significant difference in first-admission vs. re-entry patients regarding the proportion of CD8 positive cells and the smaller variation in CD4+ and CD8+ cells and CD4/CD8

ratios suggests that the immunological aberrations are more established among the patients with an advanced stage of the disease.

Previous studies of T lymphocyte subsets in schizophrenia have been conducted on the peripheral blood level, and they have produced a bulk of contradictory findings. The most recent PB studies have been more thoroughly planned than the previous ones, and they have produced results which support the view of immunological aberrations in schizophrenia. However, the 'immune privilege' of the brain, the communicative functions of the blood-brain-CSF barrier and previous reports on nonparallel findings of PB and CSF cells in other CNS diseases are facts that may diminish the reliability of immunological data obtained on the peripheral body compartment. Viewed from this angle, the finding of lymphocyte deviations in the CSF of schizophrenic patients suggests more clearly that immunological abnormalities do occur in connection with schizophrenia. Our finding of no association between the patients' CSF and PB lymphocyte findings further emphasizes the superiority of CSF studies over PB analyses in schizophrenia and other CNS diseases.

9.3. The quantitative and proportional cytology of CSF mononuclear cells

The distributions of mononuclear cells in the CSF from schizophrenic patients in **Study II** were almost invariably skewed from lymphocyte domination to macrophage enrichment. This is a frequent finding in CNS injuries and degenerative CNS diseases, usually accompanied by an increase in the total number of CSF cells. The absence of pleocytosis and heavy lipophages in the patient samples of this study make this macrophage accumulation more susceptible to subtle chronic or subchronic degenerative processes than acute neuronal injuries within the CNS. This finding lends some support to the hypothesis of slight progressive brain substance loss in the longitudinal course of schizophrenic psychosis which have been proposed on the basis of several neuroradiological studies of this illness.

The majority of macrophages detected in the CNS and CSF are considered to be of microglial derivation (Thomas 1992). This relationship makes the finding of this study potentially important also from the neurodevelopmental point of view. The microglia network is formed from the hematopoietic stem cells during fetal development, and various pathophysiological events within the CNS are able to induce the activation and mobilization of these resting cells into pluripotent immunocompetent scavengers and defenders with HLA-DR expression and ability to act as antigen-presenting cells, to have phagocytic capacity, and to produce cytokines and neurotoxins. The CSF

cell composition with macrophage dominance over lymphocytes is also typical in newborn infants. This further tempts one to speculate that this finding may be in connection with cells of the macrophage-microglial lineage participating in the pathophysiology of schizophrenia during CNS development, or may reflect a genetically transformed immune response to environmental factors.

9.4. Morphological characteristics of CSF lymphocytes and monocytes

From the early '60s on, the appearance of atypical lymphocytes in blood smear samples from schizophrenic patients has been a sporadic topic of discussion in the field of schizophrenia research. This important but debatable finding was introduced by Fessel and Hirata-Hibi, and during the following three decades Hirata-Hibi has occasionally repeated and defined the presentation of 'P cells'. One argument against the 'P cell' has been the fact that it has been the finding of one research group, and there have been no other reports on these cells. The morphological analysis of MGG-stained cytocentrifuged CSF slides from schizophrenic patients (**Study III**) confirm the findings of Hirata-Hibi et al. concerning the morphologically deviant lymphocytes in schizophrenia. The appearance of such cells on both sides of the BBB brings up the question of their origin and relationship to the BBB condition and function. In our study, no protein exudation was detected, which excludes the possibility of significant BBB damage and passive leakage of cells from one body compartment to another. On the other hand, active transport by transendothelial migration is conceivable, since the activation of lymphocytes tends to increase their mobility and to induce upregulated expression of e.g. adhesion molecules which contribute to cell traffic through the BBB. The rosette formations between lymphocytes and macrophages detected in this study indirectly point towards increased lymphocyte adhesiveness, and there are recent reports on elevated expression of leukocyte adhesion molecules in schizophrenia. The primary source of morphologically aberrant lymphocytes in schizophrenia is questionable. In this study, the proportions of activated and atypical lymphocytes in the CSF exceeded those in PB, but this does not necessarily demonstrate the intrathecal production of this cell line. Further studies of cell migration and BBB functions are needed to elucidate this question.

The morphological similarity of 'P cells' and CD5+ lymphocytes has been suggested by McAllister et al (1989) in connection with the detection of elevated levels of CD5+ B cells in PB from a subgroup of schizophrenic patients whose disease was thus considered to have an underlying autoimmune and/or genetic cause, because populations of these cells have been shown to be elevated in autoimmune disorders such as rheumatoid arthritis, progressive systemic sclerosis, and

Sjögren's syndrome. Recently, Printz et al (1999) have replicated the finding of elevated levels of CD5+ B lymphocytes in schizophrenia. An additional interesting feature of CD5+ cells is related to their behavior during ontogenic sequences: the preliminary forms of these cells are transferred from bone marrow to the retroperitoneal space very early, and they form the main part of B lymphocytes during fetal development, but after birth their proportion is normally reduced to less than 5%. This phenomenon may link CD5+ cells to the concept of the neurodevelopmental disorder. The morphological relationship between CD5+ cells, 'P cells' and the activated lymphocytes from CSF detected in this study requires future studies of the characterization of CSF lymphocytes by using activation markers, in order to find out whether these cells can be considered as candidates for cytological derivatives of neurodevelopmental or neurodegenerative processes during the pathogenetic cascade of schizophrenia.

The stability of the aberrant lymphocyte finding in the CSF from schizophrenic patients in the course of neuroleptic treatment and clinical improvement may signify that this could be more a trait than a state marker of the disease, and link it to the genetic background of schizophrenia. Previous detections of 'P cells' in the PB from relatives of schizophrenic patients lend support to this view.

9.5. Inflammation markers

The negative findings concerning the inflammation markers neopterin and MIP-1 α (**Study IV**) suggest, that inflammatory processes during an acute psychotic episode are not responsible for the brain substance loss which has previously been demonstrated neuroradiologically in schizophrenia. Neuronal degeneration due to inflammation is the basic pathophysiological event in some, but not all, CNS diseases, and the specific features of CNS immunological dynamics are probably part of the dilemma of brain substance changes without gliosis which is included in the complex of schizophrenia. The rapidly increasing knowledge about programmed cell death with apoptosis and dendritic pruning may offer better explanations for the immunological aberrations detected in schizophrenia.

The lack of evidence of inflammatory processes in the CNS of schizophrenic patients diverts attention from conventional immunological reactions towards more complex sequences of apoptosis and dendritic pruning in the pathophysiology of this disease. Preliminary findings of elevated concentrations of free ICAM-5 (telencephalin) in the CSF of a subgroup of schizophrenic patients

with parallel high frequencies of macrophages (Nikkilä et al *unpublished data*) lends support to this view.

10. SUMMARY AND CONCLUSIONS

A series of studies was undertaken to elucidate the role of immunocompetent cells in schizophrenia by analyzing the mononuclear cell counts and distributions, as well as T lymphocyte subsets in the CSF samples of first-admission and re-entry patients in the acute phase of the illness. Also the concentrations of inflammation markers neopterin and MIP-1 α were measured in order to clarify the possible inflammatory mechanisms in the pathophysiology of schizophrenia.

The finding of the enrichment of mononuclear cells from the mononuclear phagocyte / macrophage lineage in the CSF of schizophrenic patients suggests that mobilization of microglia is a pathophysiological event that takes place during the schizophrenic process. This novel observation may provide support to the neuroradiologically demonstrated loss of brain substance in schizophrenia, and help to explain the subtle progressive brain transformation without marked gliosis, which is still one of the most controversial topics in schizophrenia research.

The appearance of lymphocytes with atypical morphological features in the CSF reinforces the previous interesting but disputable detection of lymphocyte abnormalities in schizophrenia. The constancy of this finding, regarding the duration of the illness or the medicational status of the patients, indicates that it is a marker of a trait rather than of a state in schizophrenia, and has thus potential to be a susceptibility marker of the disease.

T lymphocyte subsets appear to display wide-range divergency in the CSF of schizophrenic patients in the initial phase of the illness, and a tendency towards CD8⁺ dominance in the group of patients with a more advanced form of the disease. This may signify an unspecific immunological imbalance due to stress factors, meningeal irritation, etc. Also diffuse traffic of these cells across the BBB, induced by macrophages / microglial mobilization, is a possible explanation for this finding. However, the significance and specificity of this finding in the pathophysiology of schizophrenia remain open until the methodology is developed sufficiently to ensure the reliability of flow cytometric investigations of CSF cells.

The negative findings regarding the intrathecal inflammatory activity measured by two sensitive inflammation markers strengthen the view that the detected aberrations of immunocompetent cells are not a reflection of conventional immunological reactions with an inflammatory response taking

place in the acute or subacute phase of schizophrenia. This tempts one to speculate that the neuropathology of schizophrenia may be based on deviant sequences of programmed cell death (apoptosis and dendritic pruning) during the neuronal development of genetically susceptible individuals.

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